Effect of Presence of Pesticides in Growth Medium on the Exudation Behaviour of Plants: A Study with Phorate (An Organophosphate Insecticide) and Pisum Sativum (L.)

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***Abstract*—** In the preset investigation, the effect of presence of phorate in the growth medium on the chemical composition of root exudates of Pisum sativum collected by two different methods was studied. Results indicated significant increase in the total protein, free thiol gruops, riboflavin, ascorbic acid, total glutathione content in the root exudates the plants exposed to phorate. Among some antioxidative and hydrolytic enzyme activities estimated, significant increase was observed in acid phosphatase, phosphodiestearse, glutathione S transferase and catalase activities in plants grown in presence of phorate with respect to controls. On the contrary, no significant changes were observed in the total carbohydrate content, organophosphate hydrolase, ascorbate peroxidase; amino acids, sugars, and phenolic acid composition. hence, results of the present investigation provide some limited by preliminary evidence on the fact that the exudation behavior of roots is altered on sensing the presence of contaminants (phorate) in the rhizosphere, which can be one of the reasons for the rhizosphere effect i.e enhance degradation of toxic pollutants in the rhizosphere.

***Keywords*—** Root exudates; Pisum sativum; Phorate; Contaminants; Rhizodegradation

I. Introduction

LITTLE attention has been given to the study of exudation behaviour of plant roots in stress conditions like the presence of toxic pollutants in the soil. Root exudates are responsible for a wide array of interactions going in the rhizosphere like plant-microbe and plant-plant. Response of plants with respect to exudation on the presence of contaminants is not yet studied. Therefore, in this study an initial attempt has been made to investigate the effect of presence of phorate, on organophosphate insecticide on the composition of root exudates of B. juncea. Pesticides are widely used in agriculture and insect vector control programs, as around 4 million tons of pesticides are applied to crops annually round the world (Miller, 2004). Organophosphorus compounds account for around 38% of total pesticides used globally (Post, 1998) and phorate {O, O-diethyl S-[(ethylthio) methyl] phosphorodithioate} is one of the high toxicity organophosphate compound extensively used to control insects (Gallo and Lawryk, 1991) on various field crops. document is a template for *Word (doc)* versions. If you are reading a paper version of this document, so you can use it to prepare your manuscript.

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II. Materials and Methods

A. Collection of root exudates

Technical grade phorate, purity >95% was provided by Meerut agrochemicals limited, India. All other chemicals were of analytical reagent grade or of a higher purity (Merck, India). Root exudates from *Pisum sativum* (L) (Sweet Pea) were collected by sand culture method (method 1) according to Gaidamak, (1971) and by extracting roots in calcium chloride (method 2) according to Steingrobe et al. (2002). Root exudates were concentrated 100 folds by lyophilization, purified by dialysis and stored at -4 ºC until further analysis (Dundek et al., 2011).

B. Analysis of the root exudates

Root exudates were analyzed for total carbohydrate content using Anthrone's method, proteins by Bradford's test, and total free thiol groups by Ellman's test (Kuwata et al., 1982). Free amino acids, sugars and organic acids were determined by thin layer chromatography (TLC) using silica gel plates. Butanol: glacial acetic acid: water (4:1:1) was the mobile phase for amino acids and 2% ninhydrin in acetone was used as developer. For sugars, chloroform: methanol: 0.25% potassium chloride (KCl) (5: 4: 1) was the solvent and orcinol (1%) in 5% H2SO4 was the indicator solution used. Analytical grade rhamnose, xylose, maltose, sucrose, raffinose, glucose, mannose, fructose, ribose, lactose, galactose were used as standards. Organic acids were detected on silica gel 60-F245 (Merck) plates using analytical grade acetic acid, butyric acid, salicyclic acid, dihydroxy benzoic acid, lactic acid, 2-ketoglutaric acid, malonic acid, oxalic acid, tartaric acid, citric acid, sulphanic acid, maleic acid as standards. The solvent system used as mobile phase for monocarboxylic acid was composed of propanol: ammonium hydroxide (7: 3) and that for dicarboxylic acids was propanol: ammonium hydroxide:

water (6: 2: 2). Bromocresol green was used as developing agent.

Activities of various antioxidative enzymes like ascorbate peroxidase, catalase, glutathione reductase, glutathione S transferase and glutathione (GSH) and hydrolytic enzymes like phosphomonoesterase, phosphodiesterase and phosphotriesterase were determined according to Rani et al. (2012). Riboflavin was detected using spectrophotofluorometer at 460-nm activation wavelength and 530-nm emission wavelength and ascorbic acid was determined by titrimetric method according to official methods of analysis of the association of official analytical chemist. Phenolic acid in root exudates were detected by high performance liquid chromatography (Waters Delta Prep, preparative chromatography system with PDA detector, Waters) with a photodiode array detector. All the samples diluted appropriately in HPLC grade methanol and filtered through a 0.2 µm membrane filter. The compounds detected were identified by the comparison with their retention times with those of pure standards. The HPLC system consisted of a Waters pumping system, PDA detector and software. Chromatographic separations were performed on a C18, column (250 × 4.0 mm ID, 5 um) and a gradient elution program with two solvents (Tian et al. 2009). Analytical grade gallic acid, vanillin, ellagic acid, caffeic acid, gentisic acid, syringic acid, p-coumaric acid, ferulic acid, benzoic acid, salicyclic acid, chlorogenic acid, phytic acid were used as standards.

III. Results And Discussion

A. Total carbohydrates, proteins and free thiol groups

The quantity of compounds present in the root exudates greatly varies with the factors like plant species, its age and method of collection (Wadhwa and Narula, 2012). No significant change ($P>0.05$) in the carbohydrate content of the root was observed between control and experimental samples (Table 1). The value of carbohydrate content varied significantly in the root exudates collected by two methods. Higher value was obtained in all the samples collected by sand culture method as compared to that by calcium chloride extraction. The differences in results of samples collected by two methods indicate that the quantity and elements of the same differ with the method for its collection. The chemical composition of root exudates is not only dependent on the plant age, species, type of soil, climatic and geographical conditions and stress, but also on the method of its collection and analysis (Shaw and Burns, 2004). Protein and free thiol content of root exudates was found to increase remarkably and significantly ($P<0.05$) in response to phorate, irrespective of the method employed for their collection as presented in Table 1. Direct involvement of proteins in phytoremediation of inorganic contaminants including heavy metals and radionuclides have been demonstrated by many workers (Gleba et al., 1999) but their role in phytodegradation of organic contaminants is seldom reported. P. sativum has been reported to enhance the biodegradation/ remediation of organic and inorganic pollutants in soil (Liste and Prutz, 2006; Tariq and Ashraf, 2013); also role of extracellular proteins exuded from roots in prevention from pathogens have been demonstrated (Wen et al. 2007). Thiol groups in root exudates may be contributed by many biological compounds like amino acids (cysteine, cystine), peptides (GSH), phytochelatins, thionate compounds, etc. Generally, thiol groups are generated by reduction of disulfide bonds by light and/or chemical activity; and a number of physiological activities have been associated with the thiol content in the plants and their levels are highly regulated by various cellular activities (Rao et al., 1983).

B. Amino acids, sugars and organic acids

Amino acids and organic acids found exudates are listed in Table 1. Out of the 11 sugars, none was detected in any of the samples. Proteinogenic amino acids released into the rhizosphere have been suggested to have some role in the direct acquisition of nutrients by plants. Release of free amino acids by plant roots has also been associated with the nutritional requirements of the rhizosphere microflora, nitrogen in particular (Odunfa et al., 1979), which causes selection and thus predominance of particular microorganisms near the root surface (Jones et al., 1994). Also, the exudation of amino acids can be enhanced by phosphorus stress and has been suggested as a mechanism to enhance mycorrhizal association (Ratnayake et al., 1978). Release of organic acids causes decline in the soil pH thus favouring the solubilization of unavailable phosphorus, consequently turning it available to plants. Secretion of organic acids in response to metal nutrient deficiency like iron (Fe) is also well documented (Kozdroj and Elsas, 2000). Presence of heavy metals (mostly toxic) in the rhizosphere and low availability of phosphorus in soil is reported to cause enhanced secretion of acids from root exudates (Pearse et al., 2006).

C. Enzymes

Activities of various enzymes in root exudates of P. sativum are listed in Table 2. the present study, the acid phosphatase activity of exudates (collected by sand culture method) was 70% higher in response to phorate as compared to controls, whereas in exudates collected by CaCl2 extraction method no significant difference was observed. Acid phosphatase activity in root exudates is regulated by the availability of plant assimilable forms of phosphorus in soil (Tarafdar and Marschner, 1994). In scarcity of available phosphorus, plants are known to secrete high amounts of the enzyme which facilitates the conversion of unavailable phosphorus to available ones; the latter is then easily taken up by the plants. Yet another fact to be noticed here is, this enzyme causes breakdown of complex organic phosphorus compounds and release of this enzyme from root exudates can be considered as an mechanism by which plants tend to attack them (phorate in this case). Exudation of phosphodiesterase enzyme from the roots of plants is a less frequently reported phenomenon; however its release from roots of certain plants has been reported (Asmar and Gissel-Nielsen, 1997). In the present investigation, the phosphodiesterase activity of the root exudates was found to increase significantly ($P<0.01$) on phorate exposure, irrespective of the method used for their collection (Table 2). Root exudate samples were also analyzed

for the presence of organophosphate hydrolase enzyme activity. Though, the samples showed the activity, no modulation in the enzyme activity could be observed on exposure to phorate. This is an unexpected observation, because the plants were shown to have potential for phorate degradation, in our earlier studies. Presence of OPH in few plants capable of degrading organophosphorus pesticides like malathion, demeton-S-methyl, and crufomate has been demonstrated (Gao et al., 2000).

Total glutathione content of the root exudates collected by both the methods was found to be significantly high ($P<0.05$) in samples with phorate exposure. High levels of GSH in roots have been reported in response to the presence of heavy metals in the soil as it acts as precursors for phytochelatins synthesis. However, inflection in the total GSH content in root exudates following exposure to organic pollutants is not yet reported, to the best of our knowledge. Taking into account, the protective role of GSH in detoxification/phytodegradation of organic contaminants, it may be hypothesized that it is secreted in high concentrations from the plant roots in response to the presence of phorate in the rhizosphere. Enzyme activities like glutathione S transferase (GST), ascorbate peroxidase (APX) and catalase (CAT) could be detected only in the samples collected by sand culture and none of the enzyme activities were observed in exudates collected by CaCl2 extraction. GST and CAT activities were found in increase significantly ($P>0.05$) whereas no significant change ($P>0.05$) was observed in APX activity of the control and experimental root exudate samples. Increment in the activities of antioxidative enzymes like SOD, APX and glutathione reductase (GR) when grown in soil contaminated with inorganic contaminants have been reported (Pandey and Singh, 2012; Malecka et al. 2012).

D. Vitamins (Riboflavin & Ascorbic acid), Phenolic acids

Riboflavin content of the root exudates (both the methods) of P. sativum was high in response to phorate ($P<0.05$) as evident from Table 1. Release of riboflavin from plant roots is well reported. Its secretion by plants is reported to be induced by Fe deficiency (Ohwaki and Sugahara, 1997; Welkie, 2000). Release of riboflavin by plant roots also have important role in plant-microbe interactions. Some riboflavin in the root zone is degraded to lumichrome, which stimulates root respiration (Phillips et al., 1999) and may, therefore, contribute to the exogenous CO2 required for growth by this organism (Lowe and Evans 1962). In this way riboflavin in root exudates play significant role in plant-microbial interactions. Results of the present study showed that ascorbic acid was exuded from the roots of B. juncea and the concentration was significantly high ($P<0.05$) in experimental samples as compared to control. Exudation of ascorbic acid from plant roots have been demonstrated by several workers (Tu et al. 2004). Its exudation by plant roots has been associated with metal (nutrient) chelation, phosphate acquisition, and antioxidative effect.

Phenolic acids commonly act as nutrient source for the microflora of soil. Many of them have been reported as chemoattractant signals to microbes, microbial growth promoters, nod gene inducers and inhibitors in rhizobia. As most of them carry carboxyl functional group they act as chelators of poorly soluble mineral nutrients, thereby making them available to plants. Many phenolic compounds are important in plant's defense against pathogens and act as phytoalexins and have allelophathic functions (Tang and Young, 1982). Thus, phenolic acids are important chemicals in plant-microbe interaction. Secretion of phenolic compounds from plant roots have also been frequently reported under iron and phosphorus deficient conditions (Jin et al., 2007).

IV. Conclusions

Hence, on the basis of our observations we may conclude that the composition of root exudates of P. sativum, contain a wide array of chemical compounds which have different physiological functions in nature. Also, it was observed that the presence of chemical contaminants like phorate in this case effects the chemical composition of both root exudates and chemical composition differ with the method of collection. Change in the total protein, thiol content and enzyme activities on phorate exposure suggest the same. In absence of much literature and on the basis of experimental evidence of current investigation, we may speculate that this change in the exudation behaviour of plant roots in presence of contaminants in the growth medium may be one of the mechanisms of plant defense, particularly if the contaminant is toxic the plant and/or associated beneficial micro-flora. Due to great diversity of organic compounds released from roots and their physiological functions, it is extremely difficult to rationally conclude on the function of each and every component of the root exudates with respect to removal of contaminants. Though, there are few speculations, the exact role of each component of the root exudates on microbial population and thus removal of pollutants requires further research.

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TABLE 1.
CHEMICAL CHARACTERIZATION OF ROOT EXUDATES OF *PISUM SATIVUM* IN PRESENCE AND ABSENCE OF PHORATE

S. No	Sample → / Chemical Component ↓	M1C	M1E	M2C	M2E
1.	Carbohydrate content (mg L^{-1})	1314.5±65	1492±66	356.0±35	372.4±23
2.	Proteins (mg ml^{-1})	0.0382±0.005	0.3123±0.04	0.222±0.03	0.253±0.04
3.	Free thiol gropus (µM)	0.026±0.02	0.228±0.11	0.021±0.01	0.215±0.07
4.	Riboflavin (µg ml^{-1})	2.21±0.65	3.18±0.15	1.04±0.65	1.52±0.56
5.	Ascorbic acid (mg ml^{-1})	0.620±0.17	0.868±0.19	0.983±0.12	2.108±0.21

Note: C= without phorate; E= with phorate; M1= Method 1 (sand culture method); M2= Method 2 ($CaCl_2$ extraction method)

TABLE II
CHARACTERIZATION OF ROOT EXUDATES OF *PISUM SATIVUM*

S No	Sample → / Chemical Component ↓	M1C	M1E	M2C	M2E
1.	Amino acids	Lysine, Threonine, Tyrosine, Methionine	Lysine, Threonine, Tyrosine, Methionine	Serine, Lysine, Threonine, Cysteine, Methionine, Tyrosine, Glutamic acid, Aspartic acid	Serine, Lysine, Threonine, Cysteine, Methionine, Tyrosine, Glutamic acid, Aspartic acid
2.	Sugars	----------	----------	----------	----------
3.	Organic acids	Butyric acid, Tartaric acid, 2 ketoglutaric acid	Butyric acid, Tartaric acid, 2-ketoglutaric acid	Tartaric acid, 2-ketoglutaric acid	Tartaric acid, 2-ketoglutaric acid
4.	Phenolic acids	Caffeic acid Coumaric acid Gentisic acid Gallic acid	affeic acid Coumaric acid Gentisic acid Gallic acid	-------	---------

Note: C= without phorate; E= with phorate; M1= Method 1(sand culture method); M2= Method 2 (CaCl2 extraction method)

TABLE III
ENZYME ACTIVITIES IN ROOT EXUDATES OF *PISUM SATIVUM*

Sno	Sample → / Enzymes ↓	M1C	M1E	M2C	M2E
1.	Acid Phosphatase activity (U)	55.06±6.8	94.67±5.3	18.97±4.3	21.05±3.2
2	Phospphodiesterase activity (U)	14.88±3.4	20.83±5.3	8.93±1.9	20.83±2.6
3	OPH activity (U)	1407±56	1341±65	128.2±23	125.5±19.5
4	GSH content (nM/ml)	2.92±0.54	3.72±1.2	1.079±0.98	2.131±0.9
5	GST (U)	132.04±22	276.67±32	---------	-----
6	APX (U)	0.022±0.04	0.0188±0.01	----------	----
7	CAT (U)	3.21±0.31	16.90±4.2	--------	----

U=Units; OPH=organophosphorus hydrolase; GSH=glutathione;
GST=glutathione s transferase; APX=ascorbate peroxidase; CAT=catalase
Exudate = root exudates; Extract= Root extract; C= control (in absence of phorate); E= Experimental (in presence of phorate);
M1= Method 1 (sand culture method); M2= Method 2 ($CaCl_2$ extraction method)